Analysis of Notch1 Function by In Vitro T Cell Differentiation of *Pax5* Mutant Lymphoid Progenitors[1]

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Signaling through the Notch1 receptor is essential for T cell development in the thymus. Stromal OP9 cells ectopically expressing the Notch ligand Delta-like1 mimic the thymic environment by inducing hemopoietic stem cells to undergo in vitro T cell development. Notch1 is also expressed on *Pax5$^{-/-}$* pro-B cells, which are clonable lymphoid progenitors with a latent myeloid potential. In this study, we demonstrate that *Pax5$^{-/-}$* progenitors efficiently differentiate in vitro into CD4$^+$CD8$^+$ $\alpha\beta$ and $\gamma\delta$ T cells upon coculture with OP9-Delta-like1 cells. In vitro T cell development of *Pax5$^{-/-}$* progenitors strictly depends on Notch1 function and progresses through normal developmental stages by expressing T cell markers and rearranging *TCRβ*, γ, and δ loci in the correct temporal sequence. Notch-stimulated *Pax5$^{-/-}$* progenitors efficiently down-regulate the expression of B cell-specific genes, consistent with a role of Notch1 in preventing B lymphopoiesis in the thymus. At the same time, Notch signaling rapidly induces cell surface expression of the c-Kit receptor and transcription of the target genes *Deltex1* and *pre-Tα* concomitant with the activation of *TCR* V_β germline transcription and the regulatory genes *GATA3* and *Tcf1*. These data suggest that Notch1 acts upstream of *GATA3* and *Tcf1* in early T cell development and regulates $V\beta$-$DJ\beta$ rearrangements by controlling the chromatin accessibility of $V\beta$ genes at the *TCRβ* locus. *The Journal of Immunology*, 2004, 173: 3935–3944.

The pluripotent hemopoietic stem cell (HSC)[5] regenerates all blood cell types throughout life by differentiating via progenitor cells with increasingly restricted developmental potential. An early step in hemopoiesis is the commitment of multipotent progenitors to either the lymphoid or erythromyeloid lineages. Activation of the *RAG1/2* genes characterizes the emergence of the earliest lymphocyte progenitors, which give rise to the common lymphoid progenitors with their characteristic B, T, NK, and DC cell differentiation potential (reviewed in Ref. 1). T cell development is initiated, once these lymphoid progenitors migrate from the bone marrow to the thymus and become exposed to signals of the thymic microenvironment. The earliest phase of T cell development can be divided into distinct stages according to the differential expression of CD44 and CD25 on CD4$^-$CD8$^-$ double-negative (DN) thymocytes (2, 3). The most immature progenitors reside in the heterogeneous CD44$^+$CD25$^-$ (DN1) subset, which still possesses a broad lymphoid developmental potential (3, 4). Rearrangements of the TCRβ, γ, and δ loci are initiated in CD44$^+$CD25$^+$ (DN2) thymocytes and are completed in CD44$^-$CD25$^+$ (DN3) cells, concomitant with commitment to the T-lymphoid lineage (5–7). Thymocytes with productive *TCRγ* and δ gene rearrangements adopt the $\gamma\delta$ T cell fate. Successful recombination of the *TCRβ* gene leads to expression of the pre-TCR, which acts as an important checkpoint by selecting DN3 cells into the $\alpha\beta$ T cell lineage and promoting their differentiation to CD4$^+$CD8$^+$ double-positive (DP) thymocytes (8). DP cells rearrange the *TCRα* locus, undergo negative and positive selection at TCRαβ-dependent checkpoints, and subsequently differentiate into CD4$^+$ helper, CD4$^+$ suppressor, or CD8$^+$ killer T cells (8).

Commitment of lymphoid progenitors to the T cell lineage critically depends on signaling through the transmembrane receptor Notch1 (reviewed in Refs. 9 and 10). Notch1 is one of four mammalian Notch receptors that interact with five different ligands (Jagged1, Jagged2, Delta-like1 (DL1), Delta-like3, and Delta-like4). Ligand binding initiates two successive proteolytic cleavages of the Notch receptor, resulting in the release of its intracellular domain (ICN) from the membrane. The ICN protein translocates to the nucleus, where it binds to and converts the transcription factor recombination signal sequence-binding protein (RBP)-Jκ (also known as CBF1 or CSL) from a transcriptional repressor to an activator. Known Notch targets are the T lymphoid *pre-Tα* gene coding for an essential component of the pre-TCR (11, 12), the widely expressed *HES* and *HERP* genes coding for transcriptional repressors of the basic-helix-loop-helix protein family (13, 14) as well *Deltex1* (11, 15) and *Nrarp* (16). Notch signaling is modulated by multiple extracellular, cytoplasmic, and nuclear proteins. For instance, glycosyltransferases of the Fringe protein family modify the extracellular domain of Notch receptors, which enhances signaling by Delta-like ligands, while preventing activation by Jagged proteins (17). The Deltex1 protein inhibits Notch signaling by binding to ICN, thereby interfering with the recruitment of transcriptional coactivators to the RBP-Jκ transcription factor complex (18). Finally, the nuclear ankyrin-repeat protein Nrarp prevents activation of Notch target genes by binding to the ICN/RBP-Jκ complex, which results in degradation of the ICN protein (16).

The *Notch1* gene is broadly transcribed in hemopoietic cells, while ligands of the Jagged and Delta-like protein family are highly expressed on epithelial cells of the thymus compared with the bone marrow environment (9, 10). Notch1 signaling is therefore activated, once incoming lymphoid progenitors are exposed to a high density of Notch ligands in the thymus. Notch1 has been implicated in the control of the T vs B lineage decision and early T cell development by gain- and loss-of-function experiments (reviewed in Refs. 9 and 10). Retroviral expression of the active ICN protein or the ligand Delta-like4 in hemopoietic progenitors induces thymic-independent development of immature T cells at the expense of B lymphopoiesis in the bone marrow (19, 20). In complementary experiments, conditional inactivation of *Notch1* in HSCs arrests T cell development at the earliest DN1 precursor stage, while simultaneously promoting B cell development in the thymus (21, 22). A similar phenotype was observed by conditional inactivation of the *RBP-J*κ gene in HSCs, demonstrating that Notch1 acts via RBP-Jκ to control early T cell development (23). Retroviral expression of the negative Notch signaling modifiers Lunatic Fringe, Deltex1, and Nrarp in HSCs also blocks T lymphopoiesis at the expense of B cell development in the thymus (18, 24, 25). Hence, lymphoid progenitors in the thymus adopt the default B cell fate in the absence of Notch1 signaling. Conditional inactivation in DN pro-T cells revealed another essential role for Notch1 in controlling *V*-to-*DJ* rearrangements of the *TCRβ* locus (26). Later functions of Notch1 in T cell development remain controversial and may be confounded by the expression of other, functionally redundant Notch receptors (reviewed in Ref. 10).

Until recently, T cell development could be reproduced in vitro only in complex three-dimensional fetal thymic organ cultures (27). In contrast, stem cells and lymphoid progenitors can be efficiently differentiated in vitro into B lymphocytes by two-dimensional coculture with bone marrow-derived stromal cells (such as OP9 cells) in the presence of the lymphoid cytokine IL-7 (28, 29). Importantly, stromal cells ectopically expressing the Notch ligand DL1 lost the ability to support B lymphopoiesis, but now gained the capacity to promote in vitro T cell development (30, 31). In particular, coculture of HSCs with OP9-DL1 cells activates a normal program of T cell differentiation, resulting in DP and CD8 single-positive (SP) TCRαβ$^+$ T cells as well as in TCRγδ$^+$ T cells (31–33). The availability of a simple T cell differentiation system is likely to facilitate detailed molecular analyses of early T cell development as well as of Notch signaling (33). HSCs and lymphoid progenitors are, however, available only in small amounts and, hence, their low cell number is still a limitation for the study of the earliest developmental processes initiated by Notch signaling in the OP9-DL1 differentiation system (31).

Pax5 (B cell lineage-specific activator protein) is the critical B lineage commitment factor that restricts the developmental options of lymphoid progenitors to the B cell pathway (reviewed in Ref. 1). B cell development is arrested at an early pro-B cell stage in the bone marrow of *Pax5*$^{-/-}$ mice (34). *Pax5*$^{-/-}$ pro-B cells, which can be cultured in vitro in the presence of IL-7 and stromal cells (35), retain an extensive self-renewal and broad lymphomyeloid potential characteristic of uncommitted progenitors (36, 37). *Pax5*$^{-/-}$ pro-B cells realize, however, their lymphoid and myeloid options with different efficiencies. After transfer into sublethally irradiated mice, *Pax5*$^{-/-}$ pro-B cells efficiently develop into thymocytes within 1 wk (37), whereas macrophages and granulocytes require 2–3 mo for their generation (38). Moreover, ectopic expression of myeloid C/EBPα or GATA transcription factors efficiently induces a lymphoid-to-myeloid lineage switch in *Pax5*$^{-/-}$ pro-B cells (39). These data indicate therefore that *Pax5*$^{-/-}$ pro-B cells are in vitro clonable lymphoid progenitors with a latent myeloid differentiation potential (39).

In this study, we demonstrate that ex vivo sorted as well as in vitro cultured *Pax5*$^{-/-}$ pro-B cells efficiently differentiate into γδ and DP αβ T cells upon coculture with OP9-DL1 cells in the presence of IL-7 and Flt3 ligand (Flt3L). In vitro T cell development of *Pax5*$^{-/-}$ progenitors strictly depends on Notch1 and follows the normal developmental sequence described for in vivo T lymphopoiesis. Expression analyses identified *Deltex1*, *pre-Tα*, *GATA3*, *Tcf1*, c-*kit*, and different *Vβ* segments of the *TCRβ* locus as genes that are activated by Notch signaling at the onset of T cell development. These data indicate therefore that the combined use of *Pax5*$^{-/-}$ pro-B cells and OP9-DL1 cells is ideally suited for the molecular analysis of Notch1 function and early T cell development.

Materials and Methods

Mice

Pax5$^{+/-}$ and *Notch1*$^{F/F}$ mice were maintained on the C57BL/6 background and genotyped, as described (21, 35).

FACS sorting and analysis

The following fluorescein (FITC)-, PE-, CyChrome- or allophycocyanin-coupled Abs were used for flow cytometry: anti-B220 (RA3-6B2), CD4 (L3T4), CD3ε (145-2C11), CD8 (53-6.7), CD25 (PC61), CD44 (1M7), c-Kit/CD117 (2B8), NK1.1 (PK136), TCRβ (H57-597), TCRγδ (GL3), and Thy-1.2/CD90 (53-2.1) Abs. A wide forward/side light scatter gate was used for the analysis of all flow cytometric data. Pro-B cells were sorted as B220$^+$c-Kit$^+$ cells after enrichment of c-Kit$^+$ bone marrow cells by magnetic cell sorting (MACS). Pro-T cells were defined as Thy-1.2$^+$Lin$^-$ cells by gating away Lin$^+$ cells that were stained with FITC anti-B220, NK1.1, CD4, CD8, and TCRγδ Abs. β-Galactosidase activity was detected by loading *Pax5*$^{-/-}$ cells through hypotonic shock (75 s at 37°C) with the fluorogenic substrate 5-chloromethylfluorescein di-β-D-galactopyranoside (Molecular Probes, Eugene, OR), as described (40).

Pro-B cell culture

B220$^+$ pro-B cells were sorted from the bone marrow of *Pax5*$^{-/-}$ or *Notch1*$^{F/F}$ *Pax5*$^{-/-}$ mice and cultured on γ-irradiated ST2 cells in IL-7-containing IMDM medium, as described (35).

Generation of Notch1$^{-/-}$ Pax5$^{-/-}$ pro-B cells

Notch1$^{F/F}$ *Pax5*$^{-/-}$ pro-B cells were infected with an MSCV-Cre-GFP retrovirus (provided by D. Littman, Skirball Institute for Biomolecular Medicine, New York University School of Medicine, New York, NY) before single-cell cloning of infected pro-B cells. Individual clones were screened for Cre-mediated deletion of both floxed *Notch1* alleles as well as for the loss of retroviral GFP expression. Floxed and deleted alleles were detected by PCR using the following primers: 5′-CTGACTTAGTAGGGG GAAAAC-3′ (1), 5′-AGTGGTCCAGGGTGTGAGTGT-3′ (2), and 5′-TAAAAAGCGACAGCTGCGGAG-3′ (3). A 350-bp fragment was amplified from the floxed *Notch1* allele with primer pair 1/2 and a 470-bp fragment from the deleted *Notch1* allele with pair 1/3.

T cell differentiation of Pax5$^{-/-}$ pro-B cells

In vitro T cell differentiation was essentially performed, as described (31). The control OP9-GFP cells (31) are referred to as OP9 cells throughout this work. The differentiation medium (referred to as IL-7/Flt3L medium) consisted of α-MEM medium supplemented with 10% FCS (Eurobio, Paris, France), 50 μM 2-ME, 2 mM glutamine, 10 mM HEPES (pH 7.5), 1 mM sodium pyruvate, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1% supernatant of rIL-7-secreting J558L cells (41), and 2.5% supernatant of rFlt3L-producing SP2.0 cells (kindly provided by R. Rottapel, Ontario Cancer Institute, Toronto, Canada). *Pax5*$^{-/-}$ pro-B cells, which were either directly sorted from the bone marrow or cultured in vitro on ST2 cells in the presence of IL-7 for up to 1 mo, were seeded in 2 ml of IL-7/Flt3L medium at 4×10^3 cells/well of a 24-well plate. Each well contained 5×10^4 nonirradiated OP9 or OP9-DL1 cells. At day 4 of differentiation, 1.5 ml of medium was exchanged with fresh IL-7/Flt3L medium. The differentiating cells were passaged at a ratio of 1:10 at day 7 and subsequently at a ratio of 1:4 at days 12, 16, and 20. Contaminating OP9 cells were eliminated by

filtering the lymphocytes through a 70-μm cell strainer before replating or flow cytometric analysis.

Single-cell cloning of Pax5$^{-/-}$ progenitors

A FACSVantage TSO flow cytometer (BD Biosciences, San Jose, CA) was used to sort single B220$^+$c-Kit$^+$ pro-B cells from the bone marrow of Pax5$^{-/-}$ mice into individual wells of a 96-well plate. Each well contained 200 μl of IL-7/Flt3L medium and 1.5 $\times$ 10^4 OP9 or OP9-DL1 cells, which were γ-irradiated with 15 Gy. One-half of the volume (100 μl) was replaced by fresh IL-7/Flt3L medium at day 4 and at daily intervals starting with day 7.

Semiquantitative RT-PCR analysis

Pax5$^{-/-}$ pro-B and pro-T cells were isolated from the feeder cell cultures and separated from contaminating adherent feeder cells by incubation in a new petri dish for 45 min before harvesting the suspension cells and RNA preparation, using the TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA). Reverse transcription (with random hexamers) and semiquantitative PCR were performed, as described (39), using primers that amplify cDNA across exon-intron junctions. PCR products were separated on agarose gels and visualized by ethidium bromide staining. The primer sequences are available on request.

V(D)J recombination analysis

Differentiating T cells were digested with proteinase K, and DNA was isolated by phenol extraction and ethanol precipitation. Serial DNA dilutions were analyzed for Dβ-Jβ and Vβ-DJβ rearrangements at the TCRβ locus (26) and for Vγ-Jγ and Vδ-DJδ rearrrangements at the TCRγ and δ loci (6) by PCR amplification with published primers. PCR cycle numbers were adjusted to be in the linear range, and PCR products were detected by agarose gel electrophoresis and Southern blot analysis.

Results

Expression of Notch signaling components in Pax5$^{-/-}$ pro-B cells and stromal cells

As Pax5$^{-/-}$ pro-B cells are lymphoid progenitors (39) that can efficiently differentiate into T cells in vivo (37), we wanted to investigate whether Pax5$^{-/-}$ pro-B cells can develop into T cells also in vitro in response to Notch signaling. To this end, we first analyzed the expression of different components of the Notch signaling pathway in Pax5$^{-/-}$ pro-B cells as well as in stromal ST2 and OP9 cells, which were used together with IL-7 for in vitro propagation of Pax5$^{-/-}$ pro-B cells (35). Notch signaling was furthermore induced in Pax5$^{-/-}$ pro-B cells by transferring these cells for 2 days onto OP9 cells expressing the Notch ligand DL1 (OP9-DL1) (31). cDNA was prepared from the different cell types as well as from 12.5-day-old mouse embryos and adult thymus, and was then normalized for equal expression of the control hypoxanthine phosphoribosyltransferase (HPRT) gene before semiquantitative RT-PCR analysis of transcripts coding for different Notch signaling components (Fig. 1). All transcripts analyzed are expressed in the mouse embryo, while a subset of them could also be detected in the adult thymus (Fig. 1).

The stromal ST2 and OP9 cells fail to express Notch4, but transcribe Notch1, Notch2, and Notch3 at low, intermediate, and high levels, respectively, when compared with the embryo (Fig. 1). Of all Notch ligands, only Jagged1 is expressed at a significant level in the two stromal cell lines (Fig. 1), as previously published (31). Pax5$^{-/-}$ pro-B cells express an inverse pattern of Notch receptors, with highest expression of Notch1 and lowest expression of Notch3, whereas Notch4 transcripts could also not be detected in these cells (Fig. 1). Notch ligands are not expressed in Pax5$^{-/-}$ pro-B cells, except for the presence of a low level of Jagged2 mRNA (Fig. 1). Interestingly, Notch3 and DL1 expression is weakly induced in Pax5$^{-/-}$ pro-B cells following Notch stimulation by OP9-DL1 cells (day 2; Fig. 1). The genes coding for the Notch-signaling transcription factor RBP-Jκ (42), its transcriptional coactivator MAML1 (43), and corepressor MINT (44) are constitutively expressed, suggesting that the Pax5$^{-/-}$ pro-B cells are competent to activate and repress Notch target genes. Surprisingly, however, the Notch-regulated gene Hes1 (13) is transcribed in Pax5$^{-/-}$ pro-B cells already in the absence of Delta-Notch signaling (day 0), whereas the same lymphoid progenitors fail to express the Notch target genes Hes5 and Herp2 (14) even in response to Notch activation (day 2; Fig. 1). Pax5$^{-/-}$ pro-B cells furthermore do not express transcripts for Numb and Nrarp, which are negative modifiers of intracellular Notch signaling (16, 25, 45). However, Pax5$^{-/-}$ pro-B cells express Lunatic Fringe, which is known to inhibit Jagged-mediated activation of Notch1, while potentiating Delta-induced Notch signaling (17). Together, these data suggest that the regulatory milieu of Pax5$^{-/-}$ pro-B cells is competent for Notch signaling in response to activation by Delta-like, but not by Jagged ligands.

Efficient T cell development of Pax5$^{-/-}$ progenitors in response to Notch signaling

To investigate the in vitro T cell differentiation potential of Pax5$^{-/-}$ pro-B cells, we performed single-cell cloning experiments with c-Kit$^+$B220$^+$ pro-B cells, which were directly sorted from the bone marrow of Pax5$^{-/-}$ mice. Individual Pax5$^{-/-}$ pro-B cells were deposited into single wells of 96-well plates and then cultured in the presence of IL-7 and Flt3L on OP9-DL1 cells or control OP9 cells (31). Upon coculture with OP9-DL1 cells, 63% of all Pax5$^{-/-}$ pro-B cells grew into a colony, which consisted of ~1.2 $\times$ 10^6 cells at day 18 of in vitro propagation (Fig. 2, A and B). Flow cytometric analysis demonstrated that each colony contained on average 59% of CD4$^+$CD8$^+$ DP T cells, which coexpressed TCRβ (55%) on the surface (Fig. 2, C and D). TCR$\gamma\delta^+$ cells, which constitute a second T cell lineage (3), were generated with a 5-fold lower efficiency (11%) under the same conditions (Fig. 2, C and D). The parental OP9 cells are known to support B cell development (29) in contrast to the OP9-DL1 cells (31). The cloning efficiency of Pax5$^{-/-}$ pro-B cells was 2-fold lower on OP9 cells (33%) compared with OP-DL1 cells (63%; Fig. 2A), while the colony size was 6-fold smaller at day 18 of in vitro culture (Fig. 2B). Importantly, Pax5$^{-/-}$ pro-B cells failed to differentiate into $\alpha\beta$ or $\gamma\delta$ T cells, but instead maintained their c-Kit$^+$B220$^+$ pro-B cell phenotype during coculture with OP9 cells (Fig. 2D; data not shown). We conclude therefore that Notch signaling induces efficient in vitro differentiation of ex vivo sorted Pax5$^{-/-}$ pro-B cells into $\alpha\beta$ and $\gamma\delta$ T cells.

Notch signaling restricts the developmental potential of Pax5$^{-/-}$ pro-B cells to the T cell lineage

We next studied the developmental progression of in vitro T cell differentiation by propagating sorted c-Kit^{low}B220$^+$ pro-B cells from Pax5$^{-/-}$ bone marrow as bulk culture in the presence of IL-7, Flt3L, and OP9-DL1 cells. In parallel, we cultured sorted Pax5$^{-/-}$ pro-B cells on control OP9 cells. By day 7 of in vitro differentiation, enough cells were generated for flow cytometric analysis, which was repeated thereafter at 2-day intervals (Fig. 3). Whereas the Pax5$^{-/-}$ progenitors on the control OP9 cells maintained the c-Kit^{low}Thy-1.2$^{low/-}$B220$^+$ phenotype of pro-B cells, all cells differentiating on OP9-DL1 cells lost the B cell marker B220 and expressed the T cell marker Thy-1.2 by day 7. At this time point, most differentiating cells had a c-Kit^{high}CD44$^+$CD25$^+$ CD4$^-$CD8$^-$TCRβ^-TCR$\gamma\delta^-$ cell surface phenotype (Fig. 3), which is characteristic of the T cell precursor subset DN2 (2, 3). The majority of these cells subsequently differentiated to DN3
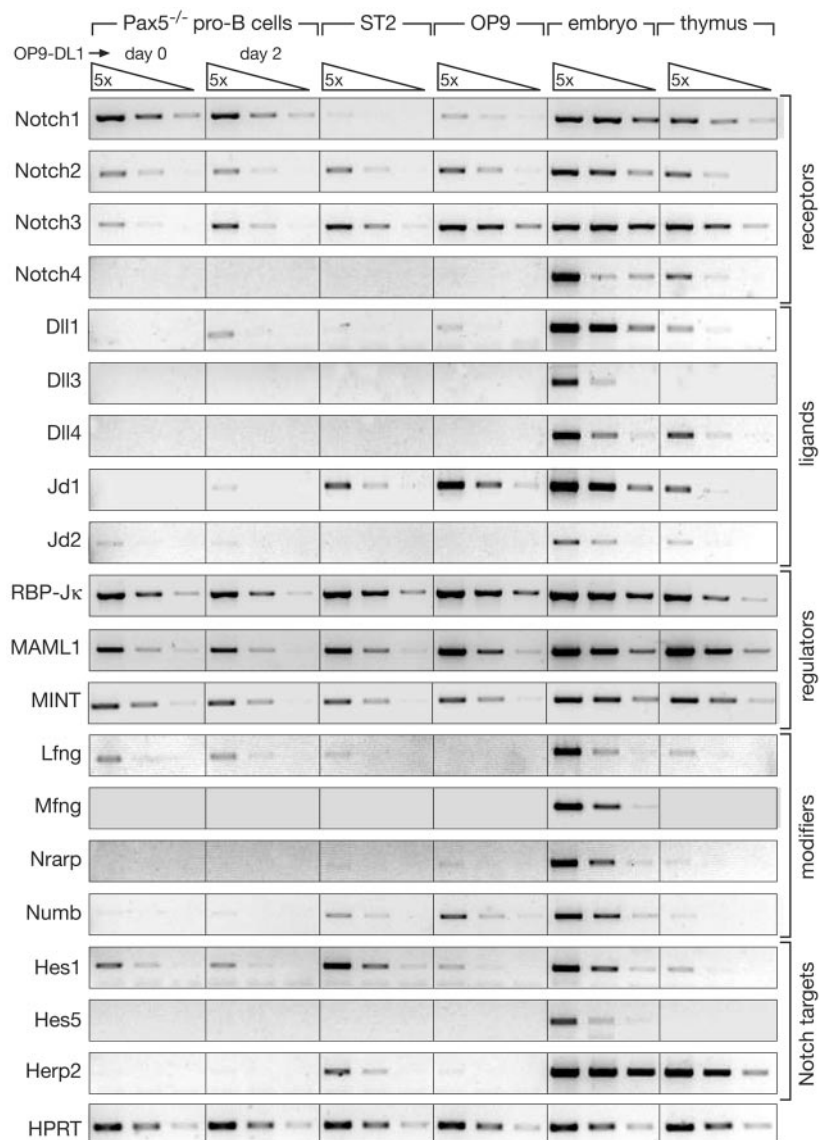
FIGURE 1. Expression of Notch signaling components in *Pax5*⁻/⁻ pro-B cells. cDNA was prepared from *Pax5*⁻/⁻ pro-B cells, stromal ST2 and OP9 cells, 12.5-day-old mouse embryos (trunk only), and adult thymus, and transcripts of the indicated genes were analyzed by semiquantitative RT-PCR of 5-fold serial dilutions of the cDNA. The cDNA input was normalized according to the expression of the control *HPRT* gene. *Pax5*⁻/⁻ pro-B cells were cultured in IL-7/Flt3L medium either on OP9 cells (day 0) or after transfer onto OP9-DL1 cells for 2 days (day 2). Dll, Delta-like; Jd, Jagged; Lfng, Lunatic Fringe; Mfng, Manic Fringe. Alternative names: MINT (SHARP), Herp2 (Hey1, Hesr1, HRT1, CHF2), and RBP-Jκ (CSL, CBF1).

cells, as indicated by the down-regulation of c-Kit and CD44 expression at days 9 and 12 (Fig. 3). DN3 cells, which have undergone functional rearrangement of the TCRβ gene, express the pre-TCR complex that signals progression to c-Kit⁻CD44⁻CD25⁻ DN4 cells and subsequent differentiation to CD4⁺CD8⁺ DP T cells (3). TCRβ⁺ DP T cells appeared at day 12 and then steadily increased until they comprised 40% of all cells at day 20 (Fig. 3). TCRγδ⁺ cells also emerged at day 12, initially increased, and then decreased relative to the TCRβ⁺ DP T cells (Fig. 3). NK1.1⁺ cells were generated only at low frequency (3.6% at day 16), whereas no Mac1⁺ myeloid cells could be detected (data not shown). In conclusion, Notch stimulation of ex vivo sorted *Pax5*⁻/⁻ pro-B cells promotes efficient T cell development at the expense of other hemopoietic lineages.

In vitro T cell differentiation of Pax5⁻/⁻ progenitors depends on Notch1

Pax5⁻/⁻ pro-B cells are known to retain an extensive self-renewal and broad developmental potential upon in vitro propagation in the presence of IL-7 and stromal ST2 cells (35–37). We therefore investigated whether in vitro cultured *Pax5*⁻/⁻ pro-B cells are also able to undergo T cell differentiation upon Notch stimulation. To this end, pro-B cells isolated from *Pax5*⁻/⁻ bone marrow were

propagated in vitro for 3 wk on ST2 cells in IL-7 medium before transfer onto OP9-DL1 cells in IL-7/Flt3L medium. On OP9-DL1 cells, the in vitro cultured *Pax5*⁻/⁻ pro-B cells down-regulated B220 expression within 7 days and efficiently differentiated into TCRβ⁺ DP and TCRγδ⁺ T cells by day 16 (Fig. 4A) similar to ex vivo sorted *Pax5*⁻/⁻ pro-B cells (Fig. 3).

Pax5⁻/⁻ pro-B cells express *Notch2* and *Notch3* (Fig. 1) in addition to *Notch1* (46). Although loss-of-function analyses have implicated only Notch1 in the control of early T cell development (21, 47), ectopic expression of the active intracellular domain of all three Notch proteins is able to induce T cell development in early hemopoietic progenitors (19, 48). To investigate whether Notch1 mediates in vitro T cell development of *Pax5*⁻/⁻ pro-B cells, we generated *Notch1*⁻/⁻ *Pax5*⁻/⁻ pro-B cells by crossing the floxed (F) *Notch1* allele (21) into *Pax5*⁻/⁻ mice, followed by in vitro culturing of *Notch1*^F/F *Pax5*⁻/⁻ pro-B cells and subsequent Cre retrovirus-mediated inactivation of the *Notch1*^F alleles. In response to IL-7 withdrawal, these *Notch1*⁻/⁻ *Pax5*⁻/⁻ pro-B cells were able to develop into macrophages with similar efficiency (data not shown) as *Pax5*⁻/⁻ pro-B cells (36). Upon coculture with OP9-DL1 cells in IL-7/Flt3L medium, the same double-mutant pro-B cells failed, however, to differentiate into TCRβ⁺ DP or TCRγδ⁺ T cell, but instead maintained expression of the B cell
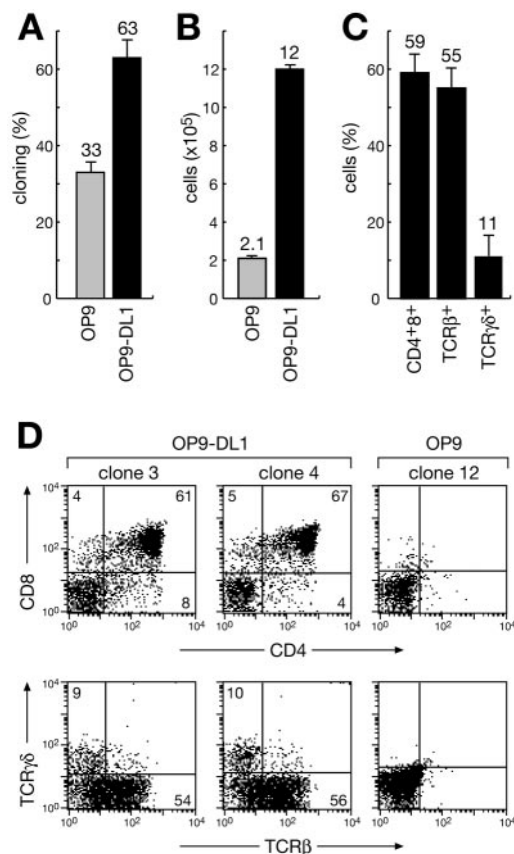
FIGURE 2. High cloning frequency and efficient T cell development of *Pax5*^{−/−} pro-B cells upon in vitro culture with OP9-DL1 cells. B220[+]c-Kit[+] pro-B cells were directly sorted from *Pax5*^{−/−} bone marrow as single cells into individual wells of a 96-well plate and cultured for 18 days in IL-7/Flt3L medium on γ-irradiated OP9 or OP9-DL1 cells. *A*, Cloning efficiency of *Pax5*^{−/−} progenitors. Three 96-well plates were evaluated. *B*, Average cell number per clone. The cells of 10 colonies were counted. *C*, Efficient T cell development of *Pax5*^{−/−} progenitors on OP9-DL1 feeder cells. Ten colonies were analyzed by flow cytometry for CD4, CD8, TCRβ, and TCRγδ expression. Average percentages of CD4^{+}CD8^{+}, TCRβ^{+}, and TCRγδ^{+} T cells are shown. *D*, Flow cytometric analysis of representative clones that were cultured in the presence of OP9 or OP9-DL1 cells. The percentages of cells in the different quadrants are indicated.

FIGURE 3. Time course of in vitro T cell differentiation of ex vivo sorted *Pax5*^{−/−} pro-B cells. Pro-B cells sorted from *Pax5*^{−/−} bone marrow were cultured on OP9-DL1 cells in IL-7/Flt3L medium for 7 days, followed by flow cytometric analysis at 2-day intervals. Expression of c-Kit, B220, and Thy-1.2 on differentiating T cells is shown by gray surface, whereas black lines denotes expression of the corresponding markers on control *Pax5*^{−/−} pro-B cells cultured in the presence of OP9 cells. CD44 and CD25 expression was analyzed on pro-T cells, which were defined as Thy-1.2^{+}B220^{−}NK1.1^{−}CD4^{−}CD8^{−}TCRγδ^{−} cells. The percentages of cells in the different quadrants are indicated.

marker B220 (Fig. 4*B*). Hence, in vitro T cell differentiation of *Pax5*^{−/−} pro-B cells is entirely dependent on Notch1 function in analogy to in vivo T cell development.

Early developmental changes following Notch stimulation of Pax5^{−/−} pro-B cells

In contrast to early B lymphopoiesis, relatively little is known about gene expression changes at the earliest stages of T cell development, as the rare thymic precursors are difficult to isolate in sufficient quantities and can furthermore not be propagated by in vitro culture (49). *Pax5*^{−/−} progenitors can, however, be grown in large quantities in vitro and, as shown in this work, are induced by Notch signaling to efficiently undergo T cell differentiation. Hence, this Notch-dependent T cell differentiation system is likely to provide novel insight into the earliest events of T cell development. We next analyzed the early changes of cell surface protein expression following Notch stimulation of *Pax5*^{−/−} pro-B cells. Expression of the T cell marker Thy-1.2 was gradually up-regulated from day 3 onward, while c-Kit expression was strongly induced already at day 1 of differentiation (Fig. 5). High c-Kit ex-
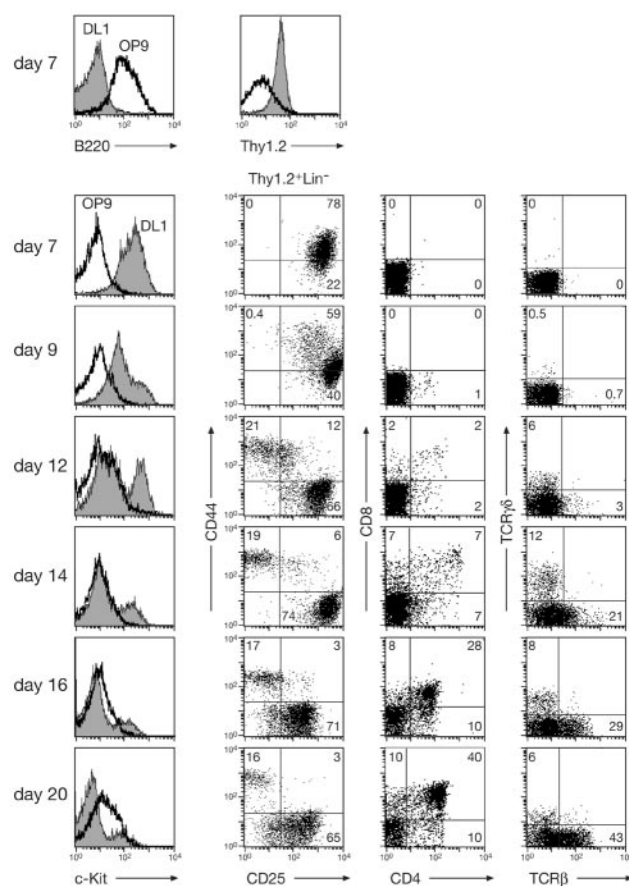
pression is a hallmark of the most immature DN1 and DN2 precursors of T cell development (3, 4, 49). The CD25 marker could, however, not be used to distinguish between DN1- and DN2-like cells, as it is already expressed on in vitro cultured *Pax5*^{−/−} pro-B cells (Fig. 5) in contrast to its absence on *Pax5*^{−/−} bone marrow pro-B cells in vivo (35). CD25 expression was, however, further increased upon coculture with OP9-DL1 cells (Fig. 5). Down-regulation of the B cell marker B220 was initiated at day 4 and was complete by day 7. Likewise, expression of the targeted *Pax5* allele was reduced already at day 3, as indicated by the loss of β-galactosidase activity (Fig. 5), which was expressed from the *lacZ* gene inserted in the *Pax5* locus (34). Hence, *Pax5*^{−/−} progenitors started to lose their B cell character and gained T cell features already 3 days after the initiation of Notch signaling.

Rearrangements of the *TCRγ* and δ loci are known to precede *TCRβ* gene rearrangements during pro-T cell development. DN2 cells undergo *Vγ-Jγ* and *Vδ-Jδ* rearrrangements and initiate *Dβ-Jβ* recombination, which is completed in DN3 cells concomitant with the onset of *Vβ-DJβ* rearrangements (5–7). By semiquantitative PCR analysis, we detected *Vγ-Jγ*, *Vδ-Jδ*, and *Dβ-Jβ* rearrrangements already in *Pax5*^{−/−} pro-B cells that were in vitro cultured on
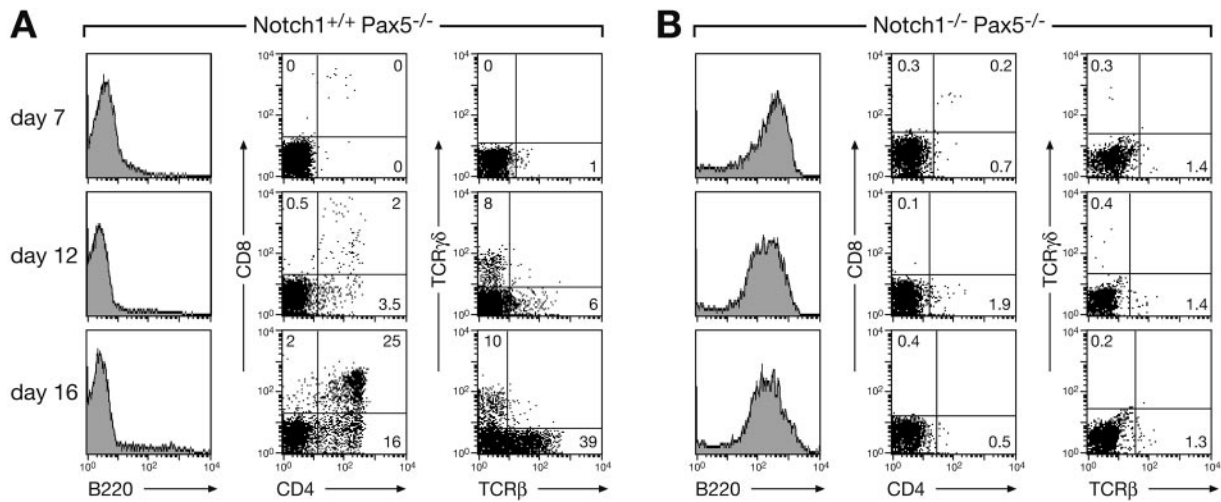
FIGURE 4. T cell differentiation of in vitro cultured *Pax5*$^{-/-}$ pro-B cells depends on Notch1. *A*, T cell differentiation of in vitro cultured *Pax5*$^{-/-}$ pro-B cells. Pro-B cells isolated from *Pax5*$^{-/-}$ bone marrow were first established in culture for 3 wk in the presence of IL-7 and ST2 cells (35) and subsequently transferred onto OP9-DL1 cells in IL-7/Flt3L medium for 7–16 days before flow cytometric analysis. *B*, Notch1 dependency of in vitro T cell differentiation. *Notch1*$^{-/-}$ *Pax5*$^{-/-}$ pro-B cells, which were derived from *Notch1*$^{F/F}$ *Pax5*$^{-/-}$ pro-B cells by Cre-mediated deletion, were cultured on OP9-DL1 cells in IL-7/Flt3L medium for the indicated days, followed by flow cytometric analysis.

control ST2 cells (Fig. 6). Whereas Notch stimulation only minimally up-regulated *Vδ5-Jδ1* recombination in differentiating *Pax5*$^{-/-}$ progenitors, it further increased *Vγ4-Jγ1* rearrangements starting at day 6 (Fig. 6*A*), when most cells were at the DN2 stage (Fig. 3). *Dβ2-Jβ2* rearrangements gradually increased with time in marked contrast to *Vβ5-DJβ2* and *Vβ8-DJβ2* recombination, which was highly active only at day 14 (Fig. 6*B*) corresponding to the DN3 stage (Fig. 3). In summary, rearrangements at the *TCRβ*, γ, and δ loci were already observed in in vitro cultured *Pax5*$^{-/-}$ pro-B cells and then increased during Notch-induced in vitro T cell differentiation in a similar temporal order as seen during pro-T cell development in vivo.

Down-regulation of B cell-specific gene expression upon Notch signaling

As *Pax5*$^{-/-}$ progenitors start to down-regulate the expression of B lymphoid proteins 3 days after Notch activation (Fig. 5), we used RT-PCR analysis to investigate the transcriptional repression of B cell-specific genes within the first 3 days after transfer of *Pax5*$^{-/-}$ and control *Notch1*$^{-/-}$ *Pax5*$^{-/-}$ pro-B cells onto OP9-DL1 cells (Fig. 7). The *Pax5*^{lacZ}, *EBF* (*early B cell factor*), λ5, *VpreB*, and *Igβ* (*B29*) transcripts were reduced at least 25-fold in *Pax5*$^{-/-}$ progenitors by day 3 of Notch stimulation, whereas transcriptional repression of the same genes did not occur in *Notch1*$^{-/-}$ *Pax5*$^{-/-}$ pro-B cells (except for a 3-fold reduction of λ5 mRNA; Fig. 7). The level of *Pax5*^{lacZ}, *EBF* (*early B cell transcription factor*), and λ5 transcripts in *Pax5*$^{-/-}$ progenitors was 5-fold reduced already within the first day of Notch activation (Fig. 7). A similar reduction of *VpreB* and *Igβ* transcripts was, however, observed only at day 2, which may reflect a longer mRNA $t_{1/2}$ or a slower kinetics of repression of these two genes in response to Notch signaling (Fig. 7). In contrast to these B cell-specific genes, Notch activation did not affect expression of the transcription factor genes *E2A* and *PU.1*, consistent with their expression during early pro-T cell development (50, 51). Interestingly, Notch signaling differentially regulated the genes coding for the tyrosine kinase receptors Flt3 and c-Kit. The *Flt3* gene was down-regulated as efficiently as the B cell-specific genes analyzed, whereas the c-*kit* mRNA levels started to increase in a Notch1-independent manner and with delayed kinetics 2 days after OP9-DL1 stimulation (Fig. 7). Hence,

the rapid increase of c-Kit protein expression at day 1 of Notch stimulation must be due to posttranscriptional regulation (Fig. 5).

The *Pax5*$^{-/-}$ pro-B cells express not only B lymphoid genes characteristic of the pro-B cell stage, but also genes of other lineage-specific programs, which are repressed by Pax5 at B cell commitment (36). Notch signaling did not affect the expression of one of these genes, *rEST1* (Fig. 7), that is expressed in thymocytes (52) (data not shown). In contrast, the *rEST2*, *rEST3*, and *J-chain* genes, which are expressed in erythroid, myeloid, and plasma cells, respectively (52) (data not shown), were 5-fold repressed within 3 days of Notch stimulation (Fig. 7). Together, these data indicate that Notch1 activation leads to a fairly rapid down-regulation of B cell-specific genes and to delayed repression of lineage-inappropriate genes in OP9-DL1-stimulated *Pax5*$^{-/-}$ progenitors.

Activation of T cell-specific genes in response to Notch signaling

Given the rapid Notch1-dependent differentiation of *Pax5*$^{-/-}$ progenitor cells into T cells, we took advantage of this in vitro system for studying the activation of early T cell-specific genes in response to Notch signaling. Previous experiments identified *pre-Tα* (11, 12) and *Deltex1* (11, 15) as putative Notch target genes that are expressed in thymocytes. Indeed, the transcription of *pre-Tα* and *Deltex1* was induced already after 4 h and continued to increase until 48 h in *Pax5*$^{-/-}$ progenitors stimulated with OP9-DL1 cells (Fig. 8). Importantly, however, Notch signaling failed to activate both genes in *Notch1*$^{-/-}$ *Pax5*$^{-/-}$ pro-B cells, thus identifying *pre-Tα* and *Deltex1* as specific targets of Notch1. The earliest phase of T cell development critically depends, in addition to Notch1, on the two transcription factors, GATA3 (53) and Tcf1 (54, 55). Interestingly, both the *GATA3* and *Tcf1* genes were also induced in a Notch1-dependent manner following OP9-DL1 stimulation of *Pax5*$^{-/-}$ pro-B cells (Fig. 8).

As Notch1 has been implicated in regulating *Vβ-to-DJβ* rearrangements (26), we next studied the chromatin accessibility of different gene segments of the *TCRβ* locus by analyzing the expression of the corresponding germline transcripts in OP9-DL1-stimulated *Pax5*$^{-/-}$ pro-B cells (Fig. 8). Notch signaling induced germline transcription of the *Vβ5* and *Vβ8* genes within 24 h,
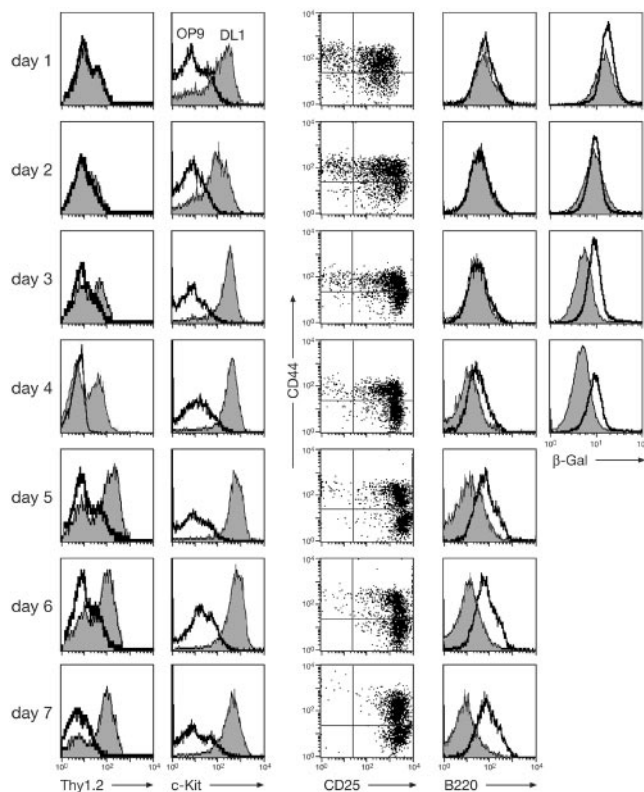
FIGURE 5. Short-term time course of T cell differentiation of in vitro cultured *Pax5*$^{-/-}$ pro-B cells. In vitro cultured *Pax5*$^{-/-}$ pro-B cells were transferred onto OP9-DL1 cells (gray surface) and cultured in IL-7/Flt3L medium for the indicated days before flow cytometric analysis. In parallel, control *Pax5*$^{-/-}$ pro-B cells were maintained in the same medium on OP9 cells (black line). The CD44/CD25 expression pattern is shown only for *Pax5*$^{-/-}$ pro-B cells cultured onto OP9-DL1 cells. Control *Pax5*$^{-/-}$ pro-B cells grown on OP9 cells exhibit the same CD44/CD25 expression profile as shown for day 1 on OP9-DL1 cells. Note that *Pax5*$^{-/-}$ pro-B cells express CD25 only upon in vitro culture (35). As the *Pax5* allele was inactivated by an in-frame *lacZ* gene insertion (34), its expression could be monitored by measuring β-galactosidase (β-Gal) activity with the fluorogenic substrate 5-chloromethylfluorescein di-β-D-galactopyranoside.

while it had only a minimal effect on the expression of *Cβ2*-containing *Dβ2* and *Jβ2* germline transcripts in *Pax5*$^{-/-}$ pro-B cells (Fig. 8; data not shown). These data suggest that Notch1 controls *TCRβ* rearrangements by regulating the chromatin accessibility at the *Vβ* gene cluster. Surprisingly, *Notch1*$^{-/-}$ *Pax5*$^{-/-}$ pro-B cells activated expression of the *Cβ2*-containing germline transcripts in response to OP9-DL1 stimulation of Notch2 and Notch3, suggesting that the *Dβ2* and *Jβ2* germline transcription is already up-regulated in response to weak Notch signaling (Fig. 8). Consequently, a low level of Notch1 signaling, which may be activated by the expression of Jagged1 on the control OP9 and ST2 cells (Fig. 1), is likely to account for the *Dβ2* and *Jβ2* germline transcripts observed in unstimulated *Pax5*$^{-/-}$ pro-B cells at day 0 (Fig. 8). In summary, our results implicate Delta-dependent Notch1 signaling in the transcriptional activation of *Deltex1*, pre-*Tα*, *GATA3*, *Tcf1*, and *Vβ* genes at the onset of T cell development.

Discussion

The initiation of T cell development entirely depends on signaling through the Notch1 receptor (21). Based on this observation, Schmitt and Zúñiga-Pflücker (31) have recently developed an in vitro T cell differentiation system, which allows hemopoietic stem cells to efficiently differentiate into αβ and γδ T cells in coculture

with stromal OP9 cells expressing the Notch ligand DL1. Notch1 is also expressed on *Pax5*$^{-/-}$ pro-B cells (46), which we have characterized as clonable multipotent progenitors with a strong lymphoid, but latent myeloid potential (36, 37, 39). In this study, we demonstrate that *Pax5*$^{-/-}$ pro-B cells efficiently differentiate in vitro into γδ and DP αβ T cells following Notch stimulation. Every second, *Pax5*$^{-/-}$ pro-B cell is able to develop into a colony consisting of ~60% DP T cells after 18 days of coculture with OP9-DL1 cells. Under these in vitro conditions, a single *Pax5*$^{-/-}$ pro-B cell gives rise to 10^6 T cells, which is similar to the estimated number of thymocytes that are generated in vivo from the earliest intrathymic progenitor (56). Importantly, in vitro T cell development of *Pax5*$^{-/-}$ progenitors is totally dependent on Notch1 function, indicating that the OP9-DL1 differentiation system faithfully recapitulates in vivo T cell development with regard to its stringent Notch1 requirement (21). Moreover, *Pax5*$^{-/-}$ progenitors upon transfer to OP9-DL1 cells rapidly down-regulate the expression of B cell-specific genes in agreement with a role for Notch1 in preventing B cell development in vivo in the thymic environment (21, 22). Notch1-stimulated *Pax5*$^{-/-}$ progenitors furthermore progress through normal T cell developmental stages by expressing T lymphoid genes and rearranging *TCRβ*, γ, and δ loci in the correct temporal sequence, which is consistent with a previous analysis of in vitro T cell differentiation of fetal HSCs (31). *Pax5*$^{-/-}$ bone marrow pro-B cells differentiate into DP T cells with similar kinetics as bone marrow-derived HSCs on OP9-DL1 cells (32). In vitro T cell development of both cell types is, however, delayed by ~5 days compared with that of fetal liver HSCs (31), thus revealing an intrinsic difference between fetal and adult hemopoietic progenitors.

Pax5$^{-/-}$ pro-B cells can efficiently be expanded in vitro on ST2 cells in the presence of IL-7 (35). In this study, we have shown that in vitro cultured *Pax5*$^{-/-}$ pro-B cells retain the potential to undergo T cell development on OP9-DL1 cells similar to ex vivo sorted *Pax5*$^{-/-}$ pro-B cells. Hence, the use of in vitro cultured *Pax5*$^{-/-}$ pro-B cells in the OP9-DL1 differentiation system allows for relatively synchronous T cell development starting with a large number of defined progenitor cells. This in vitro system is therefore well suited for the molecular analysis of early T cell progenitors, which are normally quite rare in the thymus (3, 49). In addition, this cellular system is ideal for studying the downstream effects of Notch1 signaling in early T cell development. *Deltex1*, coding for a negative regulator of Notch signaling in lymphoid progenitors (18), was previously characterized as a putative Notch target gene that is activated by ectopic expression of ICN in DP T cells (15, 57). Our finding, that the stimulation of *Pax5*$^{-/-}$ progenitors by OP9-DL1 cells rapidly induces *Deltex1* transcription in a Notch1-dependent manner, defines this gene as a specific target of Notch1 in early T cell development. Likewise, the pre-*Tα* gene coding for an essential component of the pre-TCR receptor is rapidly activated in Notch-stimulated *Pax5*$^{-/-}$ pro-B cells, consistent with the presence of functional RBP-Jκ binding sites in the pre-*Tα* enhancer (12). The *Hes1* gene also contains two functional RBP-Jκ binding sites in its promoter (13), is activated by ectopic ICN expression in DP thymocytes, and is thus thought to be a direct target of Notch signaling (11). Surprisingly, however, Notch-Delta signaling is not required for *Hes1* expression in in vitro cultured *Pax5*$^{-/-}$ pro-B cells. Two observations may be relevant in this context. First, the stromal ST2 and OP9 cells express *Jagged1* in contrast to *Delta-like* genes (31) (Fig. 1). Second, *Pax5*$^{-/-}$ pro-B cells express the glycosyltransferase gene *Lunatic Fringe*, which prevents Notch signaling by Jagged proteins, while enhancing Notch activation by Delta-like ligands (17). It is thus conceivable that incomplete inhibition of Notch-Jagged1 signaling by Lunatic

FIGURE 6. *V(D)J* recombination of the *TCRβ*, γ, and δ loci in differentiating *Pax5*^{-/-} T cells. *A*, *TCRγ* and *TCRδ* rearrangements. In vitro cultured *Pax5*^{-/-} pro-B cells were transferred from ST2 cells onto OP9-DL1 cells for the indicated time before DNA preparation. Threefold serial dilutions of the DNA were analyzed by PCR for the presence of the indicated *Vγ-Jγ* and *Vδ-Jδ* rearrrangements. Input DNA was normalized by amplification of a PCR fragment from the *Cμ* regions of the *Ig H chain* (*IgH*) locus. DNA of DP thymocytes and stromal ST2 cells were used as positive and negative controls, respectively. The gene segments of the *TCRγ* locus are numbered according to Hayday et al. (66). *B*, *TCRβ* rearrangements. *Dβ2-Jβ2*, *Vβ5-DJβ2*, and *Vβ8-DJβ2* rearrangements were detected by PCR in Notch-stimulated *Pax5*^{-/-} progenitor cells. Numbers to the *right* of the ST2 lane indicate rearrangements to the *Jβ2.1*, *Jβ2.2*, *Jβ2.3*, *Jβ2.4*, *Jβ2.5*, and *Jβ2.6* segments, respectively. GL denotes the position of the germline PCR product.
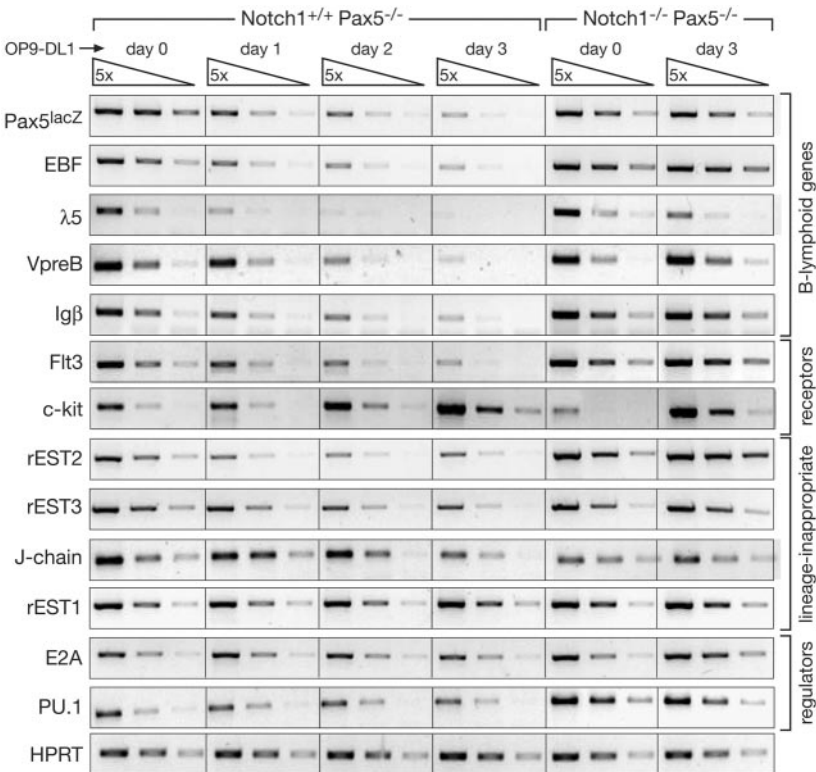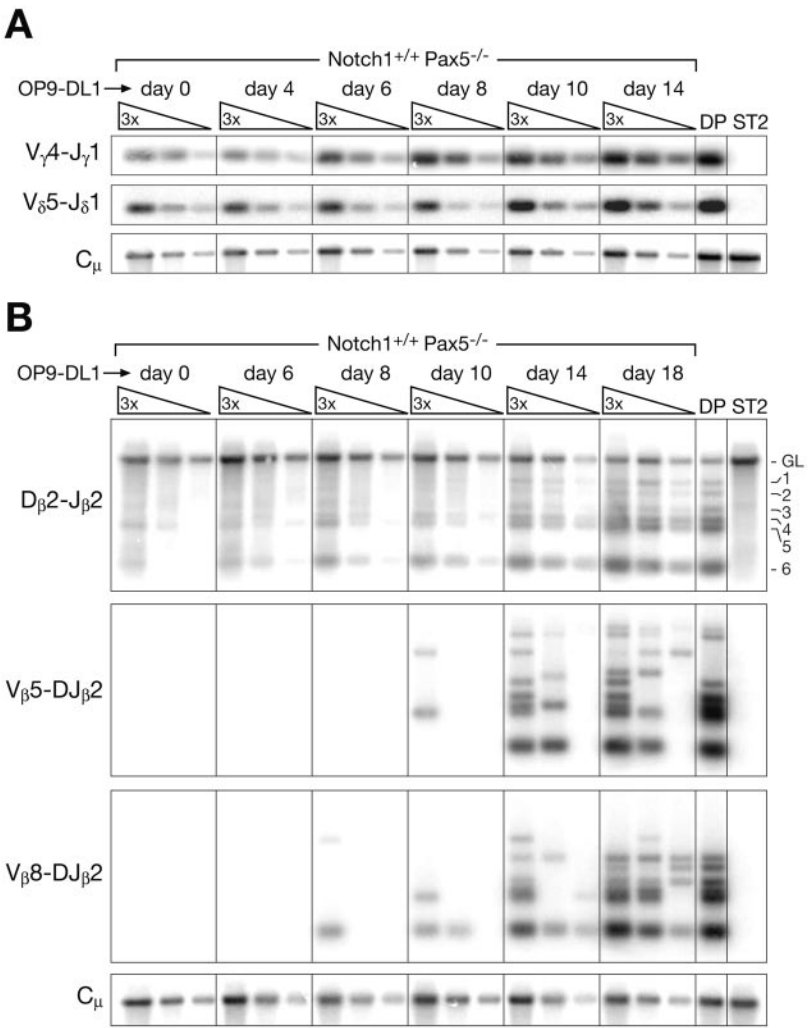

FIGURE 7. Down-regulation of B cell-specific genes upon Notch stimulation of *Pax5*^{-/-} progenitors. *Pax5*^{-/-} and *Notch1*^{-/-} *Pax5*^{-/-} pro-B cells, which were in vitro propagated on ST2 cells (day 0), were transferred onto OP9-DL1 cells and cultured in IL-7/Flt3L medium for the indicated days before RNA isolation and cDNA preparation. Five-fold serial dilutions of the cDNA were analyzed by semiquantitative RT-PCR for expression of the indicated genes. The cDNA input was normalized according to the expression of the control *HPRT* gene. Transcriptional activity of the *Pax5* locus was monitored as *lacZ* mRNA expression, as the *Pax5* gene was inactivated by an in-frame *lacZ* gene insertion (34). rEST1–3 correspond to Pax5-repressed (r) transcripts (52).

FIGURE 8. Induction of T cell-specific genes in *Pax5⁻/⁻* progenitors following Notch stimulation. *Pax5⁻/⁻* and *Notch1⁻/⁻ Pax5⁻/⁻* pro-B cells were transferred from OP9 cells (0 h) to OP9-DL1 cells and cultured in IL-7/Flt3L medium for the indicated hours before RNA isolation and cDNA preparation. The cDNA input was normalized for equal *HPRT* expression and then analyzed by semiquantitative RT-PCR for expression of the indicated genes. GLT, germline transcript.
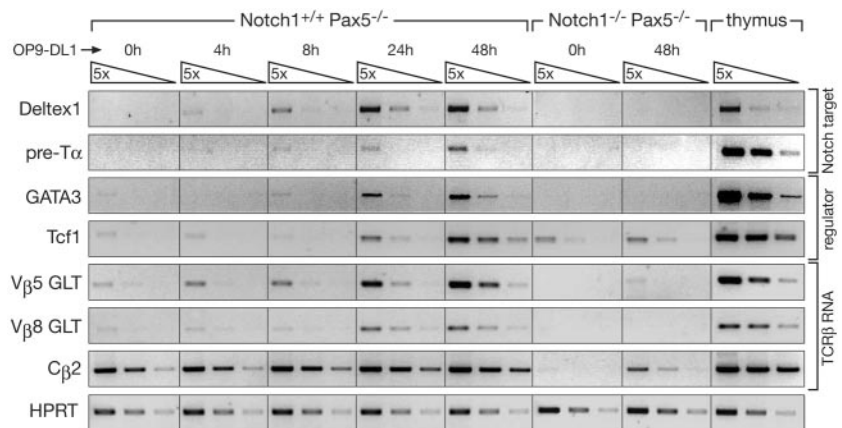
Fringe may lead to the activation of *Hes1* in cultured *Pax5⁻/⁻* pro-B cells despite the absence of Delta-like ligands. Alternatively, the *Hes1* gene is regulated independently of Notch signaling in early lymphoid progenitors, which is further suggested by its normal expression in *Notch1⁻/⁻ Pax5⁻/⁻* pro-B cells (data not shown) in analogy to the situation described in *Notch1⁻/⁻* and *RBP-Jκ⁻/⁻* embryos (58).

An interesting finding of our study is the rapid up-regulation of c-Kit protein expression on the surface of *Pax5⁻/⁻* pro-B cells within 1 day of Notch activation. Surprisingly, however, the c-*kit* mRNA levels start to increase only 2 days after Notch stimulation, demonstrating that c-*kit* is unlikely to be a transcriptional target of Notch signaling. Instead, these data demonstrate that Notch signaling controls the rapid induction of c-Kit protein expression at the posttranscriptional level. High c-Kit expression is a distinctive feature of the earliest T cell progenitors in the thymus (4), while thymus-seeding precursors in the bone marrow (59) express either no or only low levels of c-Kit on their cell surface (60). It appears therefore that the up-regulation of c-Kit expression is one of the earliest responses of thymic immigrants to the dense Notch ligand environment of the thymus.

Notch signaling functions as a molecular switch by turning the RBP-Jκ transcription factor complex from a repressor to an activator of gene expression (9, 10). In the absence of ICN, RBP-Jκ represses target genes by associating with corepressor complexes containing CIR1, SMRT, MINT, and histone deacetylases (9, 10). Upon Notch signaling, the ICN protein dissociates these corepressor proteins from RBP-Jκ and recruits coactivators together with the histone acetylases p300, PCAF, and GCN5 to the RBP-Jκ complex (9, 10). In the context of histone acetylation, it is interesting to note that Notch stimulation of *Pax5⁻/⁻* pro-B cells activates *Vβ* germline transcription of the *TCRβ* locus to significant levels within 24 h. These data therefore suggest that Notch1 controls chromatin accessibility at the *Vβ* gene cluster either directly or indirectly by inducing local histone acetylation at the onset of T cell development. *Vβ*-to-*DJβ* rearrangements of the *TCRβ* locus are, however, initiated only at day 10 of in vitro T cell development, thus confirming that an open chromatin state is necessary, but not sufficient for *V(D)J* recombination (61). A role for Notch1 in chromatin regulation of the *Vβ* genes could also explain the specific *Vβ*-to-*DJβ* recombination defect that was observed upon *lck-cre*-mediated inactivation of *Notch1* in pro-T cells (26).

Early T cell development depends, in addition to Notch1, on the transcription factors GATA3 and Tcf1. The *GATA3* gene is expressed within the hemopoietic system in HSCs, common lymphoid progenitors, and NK cells of the bone marrow (62, 63), as well as in a dynamic pattern during thymocyte development (64).

GATA3 deficiency arrests T lymphopoiesis at the earliest stage in the thymus (53), while conditional inactivation within the T lymphoid lineage has revealed novel GATA3 functions in the control of *β*-selection and in the development of CD4 SP T cells (65). *Tcf1* expression is initiated in the earliest thymocyte progenitors, and is essential for the maintenance and expansion of the DN2, DN4, and intermediate single-positive thymocyte compartments in adult mice (54). Consistent with this notion, T cell development is progressively lost with age and ultimately arrests at the early DN1 stage in adult *Tcf1⁻/⁻* mice (54, 55). In this study, we have shown that Notch1 signaling in *Pax5⁻/⁻* pro-B cells induces the transcription of both the *GATA3* and *Tcf1* genes within 24 h. Although these data do not allow us to distinguish between a direct or indirect role of Notch1 in regulating these two genes, they unequivocally demonstrate that *GATA3* and *Tcf1* act downstream of *Notch1* in early T cell development. Our analysis of Notch1 function in *Pax5⁻/⁻* progenitors has thus provided the first insight into the regulatory network controlling the earliest phase of T cell development. We are currently performing cDNA microarray screens to obtain a more comprehensive picture of the gene expression changes that are initiated by Notch signaling in *Pax5⁻/⁻* progenitor cells.

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